PERIODIC ANTIMICROBIAL PEPTIDES

PRIOR RELATED APPLICATIONS

[1] This application claims priority to U.S. Patent Application 10/373,306, filed February 24, 2003.

FEDERALLY SPONSORED RESEARCH STATEMENT

[2] Not applicable.

FIELD OF THE INVENTION

[3] The invention comprises a novel process for producing periodic peptides, as well as the peptides themselves and the use of those peptides in a variety of therapeutic applications, such as antimicrobial, antibacterial, antiviral, or anti-tumor agents and other therapeutics, disinfectives, preservatives, and the like.

BACKGROUND OF THE INVENTION

[4] Antimicrobial peptides are common weapons in the natural defense arsenal of many types of organisms, including mammals, birds, reptiles, insects, plants and many microorganisms. Naturally occurring antimicrobial peptides are unique sequences that are about 10 to 50 amino acids in length. They tend to be rich in basic amino acids (lysine and arginine) and thus cationic. They are also often amphipathic in nature (i.e., one part of the molecule is hydrophilic while the other part is hydrophobic).

[5] Although widely studied, the mode of action of antimicrobial peptides remains the subject of scientific debate. In many cases, the data suggests that the amphipathic peptides organize to form pores or channels in membranes (Durell (1992)). In other experiments, the antimicrobial peptides appear to disrupt a membrane by forming a "carpet-like" association with the membrane (Gazit (1995)). Either mechanism disrupts and kills cells by causing membrane depolarization and the loss of essential cellular components.

[6] Microbial selectivity stems from the difference between mammalian and microbial cells as to the lipid composition of the membranes. The outer leaflet of mammalian cell membranes is almost entirely composed of electrically neutral, zwitterionic phospholipids, mainly phosphatidylcholine, sphingomyelin and cholesterol. By contrast, bacterial membranes consist of mainly negatively charged phospholipids, such as phosphatidylglycerol and cardiolipin. Thus, bacterial cells are susceptible to the cationic antimicrobial peptides, while mammalian cells are not. There is also some evidence to suggest that cancer cells may also differ in their membrane components from normal mammalian cells, making tumor cells susceptible to antimicrobial peptides.

[7] Antimicrobial peptides can also be expected to have efficacy against viruses, such as HIV, herpes simplex and cytomegalovirus. However, the mechanism differs slightly. A virus is generally immune to membrane-bursting mechanisms because of the outer protein coat, but several antimicrobial peptides have shown antiviral activity by either blocking fusion of the virus with the host cell wall (thereby preventing transmission of the genetic material into the host cell) or by inhibiting replication of the virus once the host cell wall has been breached.

[8] In light of the widespread appearance of pathogens that are drug resistant, there is interest in using antimicrobial peptides as an alternative to typical small molecule drugs **if** they could be economically produced. However, a practical limitation to large-scale uses of antimicrobial peptides is that they are expensive to produce in mass quantities. For example, peptide synthesis is very costly because the peptides are of unique sequence. Each amino acid must be added to a growing peptide chain, usually with less than perfect efficiency. Thus, as chain length increases, yields decrease.

[9] The recombinant production of proteins provides some advantages over solid phase synthesis, including sequence fidelity, convenience, low cost, and the ability to produce longer proteins. However, recombinant techniques cannot be universally applied, and the recombinant production of antimicrobial peptides is particularly difficult due to their tendency to kill a variety of host cells. Even when synthesized as inactive fusion proteins, the precursor must still be cleaved to liberate the active peptide and further purification is usually required. These additional steps increase the cost and decrease the yield of the recombinant protein.

[10] Demegen, Inc. of Pittsburgh, PA owns several peptides which are being developed for medical uses. One is D2A21 (FAKKFAKKFKKFAKKFAKFAFAF) being developed under the trade name DEMEGEL.™ This unique antimicrobial peptide is an amphipathic α-helix peptide that uses groups of 4 and 3 amino acids in order to keep the polar and non-polar faces aligned (3.6 residues/turn). It is synthesized by traditional methods, one amino acid at a time.

[11] D2A21 has activity against a variety of cell types, including *T. vaginalis, C. trachomatis*, and *P. aeruginosa*. Preliminary results have also established anti-tumor activity in a rat prostate adenocarcinoma model, improving the survival rates from 25% to 75% and not causing any significant toxicities. Although uncertain of the basis for this activity, it is suggested that tumor cell membranes are substantially different from those of normal cells and therefore more susceptible to lysis by antimicrobial peptides (Arlotti (2001)). Finally, D2A21 has also been shown to have activity against the herpes simplex virus (HSV). When mixed with a modified lipid octyl-glycerol, D2A21 was better than five other peptides (including magainins and defensins) against HSV.

[12] Although very promising, peptides like D2A21 must be made one amino acid at a time, for a cost of about US $50-500/g. As another example, nisin is an antimicrobial peptide used in processed dairy products, which sells for approximately $6000/pound of active peptide.

[13] An alternative approach is to design peptides that have a several-amino acid repeat unit. The short sequence of amino acids could be synthesized less expensively than a long peptide and the repeat unit oligomerized to reach the full peptide length. Recent efforts using this approach include US5789542 and Javadpour (1996). These references teach that 7 residue (7mer) repeat units, polymerized into 14 and 21 residue peptides, can form the basis for antimicrobial peptides. By using the 7mer, a "simulated" α-helix is made, complete with an 3.5 amino acids per turn. However, the 7mer is still quite expensive to synthesize, thus limiting this approach.

[14] Desirably, a process would exist that could inexpensively produce peptides having comparable antimicrobial activity to unique peptides. More desirably, the antimicrobial peptides produced by such a process would not require adherence to the classical α-helix structure, so that small repeat units of fewer than 7 residues could be used

to construct the final peptide. By virtue of their simplicity, the peptides would be inexpensive to make, yet have significant antimicrobial activity.

SUMMARY OF THE INVENTION

[15] The invention comprises a method of producing antimicrobial periodic peptides and further comprises the peptides themselves and a wide variety of their uses.

[16] In a preferred embodiment, simple peptides are made from monomer units of four or fewer amino acids. Identical monomers units are joined end to end until a minimum size of about 15-16 amino acids is reached. By designing periodic peptides that use only tetramer (4mer), trimer (3mer) or dimer (2mer) monomers, the cost of production is reduced substantially as compared to traditional custom synthesis methods. Further, even if a given peptide were less active on a performance per dose basis, the significantly lower production cost still results in reduced cost per unit dosage.

[17] The monomers may be produced synthetically or through microbial, viral or enzymatic expression. The smaller the monomer, the lower the cost of preparation. Dimeric monomers units may be commercially available at low cost and are particularly preferred. Identical monomers may be multimerized one by one to control the ultimate size or as a mixture and then selected for size. Alternatively, mixtures of different sizes can also be employed and this is a particularly preferred embodiment.

[18] Each monomer should contain a positively charged amino acid, such as lysine, arginine, and the like. The monomers should also contain a hydrophobic amino acid, such as alanine, valine, and the like, and preferably, at least one of the hydrophobic amino acids has a bulky side chain such as phenylalanine. However, no clear trends were detectable when peptide activity was compared against hydropathy.

[19] It is preferred that at least 25% of the peptide (by number, not weight) be positively charged amino acids, and preferably at least 30%. Antimicrobial activity has been detected in periodic peptides with as much as 75% cationic residues.

[20] Preferably, the overall chain length of the resulting peptide should be at least 14 to 16 amino acids in length, but activity has been detected in peptides as small as 4 residues. An upper size limit on activity has not yet been found, but even if active, it is

expected that very large multimers will be susceptible to stability or systemic transport problems. Thus, we have suggested a practical limit of about 50, 80 or 100 residues, and preliminary results indicate that even peptides as long as 80 residues are active. Most preferably, the overall chain length of the resulting peptide is from about 14-40 or 16-36 or 20-24 amino acids in length.

[21] The peptides may contain either natural or synthetic amino acid with characteristics as described above. They may be made with either D or L amino acids. Peptides made with D amino acids have some advantage in being less susceptible to proteolytic degradation. Mixed peptides should be predominantly D (80%) in order to take advantage of this feature. Non-peptide linkages may also be employed in order to improve the stability of the "peptides." The peptides tested herein were not capped, but had free amino and carboxy termini. However, capping and derivatizing may be employed as needed.

[22] "Antimicrobial activity" means activity against bacteria, yeast, fungi, and other protozoans at a level less than or equal to an IC50 of 125 ug/ml. Anti-bacterial and anti-fungal activities are similarly defined. "Biocidal activity" means having killing activity of less than or equal to 125 ppm for 3.5 log kill at 24 hr. "Antiviral activity" means activity against viruses at an IC50 of less than 5 mM, and preferably less than 1 mM. "Anti-tumor activity" means activity against a tumor cell at a level less than or equal to a TX50 of 250 μg/mL or (50% toxic dose).

[23] Many periodic antimicrobial peptides can be made according to the general process. The general formulae for the monomers is P2N2, P3N, PN2, P2N, and NP, wherein P is any cationic residue and N is any hydrophobic residue and the N and P residues are in any order (in **all** cases the first and second P or N residues may be the same or differ within a given monomer). Preferred sequences include PNNP, NNPP, NPPN, PPNN, PNPN, NPNP, PNP, NPP, PPN, NPN, PNN, NNP, NP and PN. Preferably, the P can be any of K (lysine), O (ornathine), or R (arginine) and N can be any of A (alanine), F (phenylalanine), G (glycine), L (leucine), I (isoleucine), T (threonine), Y (tyrosine), W (tryptophan), V (valine), or M (methionine).

[24] The periodic peptides have a wide variety of applications, including agricultural (use in fields, orchards, vineyards, gardens, etc., for control of bacterial, fungal

and viral pests); post harvest grain, fruit, and vegetable treatments; veterinary use; personal hygiene products; baby products; personal wipes; hard surface disinfectants; pharmaceutical uses to treat infections and tumors; skin treatments (dandruff, acne, psoriasis); drug permeability enhancers; medical device treatments; eye treatments (infection control, contact lens disinfection, contact lens solution preservative); pharmaceutical preservatives (such as vaccines); personal care product preservation; household product preservation; food, feed processing; meat processing disinfectant; potable water, juice and beverage preservative; and food & feed preservatives. They may also be the active ingredient in liquid soaps, toothpaste; hard surface cleaners and disinfectants; bathroom and kitchen cleaners; deodorants, textile and skin treatments, and the like.

EXAMPLE 1

[25] Periodic peptides were ordered from a commercial peptide manufacturer for the initial antimicrobial tests. The antimicrobial peptides tested to date include those listed in Table 1. The ends of the peptides were not capped (free H and OH).

[26] Table 1. Specific Periodic Peptide Sequences

SEQ ID NO	SEQUENCE
1.	KFAK KFAK KFAK KFAK
2.	KFAK KFAK KFAK KFAK KFAK
3.	KFAK KFAK KFAK KFAK KFAK KFAK
4.	KFAK KFAK KFAK KFAK KFAK KFAK KFAK
5.	KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK
6.	KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK
7.	KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK
8.	KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK KFAK
9.	RFAR RFAR RFAR RFAR RFAR RFAR
10.	RFAR RFAR RFAR RFAR RFAR RFAR RFAR
11.	RFAR RFAR RFAR RFAR RFAR RFAR RFAR RFAR
12.	FAKK FAKK FAKK FAKK FAKK FAKK
13.	AKKF AKKF AKKF AKKF AKKF AKKF
14.	KKFA KKFA KKFA KKFA KKFA KKFA
15.	LKKL LKKL LKKL LKKL LKKL
16.	LKKL LKKL LKKL LKKL LKKL LKKL
17.	LKKL LKKL LKKL LKKL LKKL LKKL LKKL
18.	LKKL LKKL LKKL LKKL LKKL LKKL LKKL LKKL
19.	KFAF KFAF KFAF KFAF KFAF KFAF KFAF
20.	KFFK KFFK KFFK KFFK KFFK KFFK KFFK
21.	KFAK KFAK KFAK KFAK KFAK KFAK KFAK
22.	KAAK KAAK KAAK KAAK KAAK KAAK KAAK

23.	KKAK KKAK KKAK KKAK KKAK KKAK KKAK
24.	KFK KFK KFK KFK KFK
25.	KFK KFK KFK KFK KFK KFK
26.	KFK KFK KFK KFK KFK KFK KFK
27.	KFK KFK KFK KFK KFK KFK KFK KFK
28.	KFK KFK KFK KFK KFK KFK KFK KFK KFK
29.	KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK
30.	KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK
31.	KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK
32.	KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK KFK
33.	FKA FKA
34.	FKA FKA FKA FKA
35.	FKA FKA FKA FKA FKA FKA
36.	FKA FKA FKA FKA FKA FKA FKA
37.	FKA FKA FKA FKA FKA FKA FKA FKA
38.	FKA FKA FKA FKA FKA FKA FKA FKA FKA
39.	FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA
40.	FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA
41.	FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA FKA
42.	LK LK
43.	LK LK LK LK LK
44.	LK LK LK LK LK LK LK LK
45.	LK LK LK LK LK LK LK LK LK
46.	LK LK LK LK LK LK LK LK LK LK
47.	LK LK LK LK LK LK LK LK LK LK LK
48.	LK LK LK LK LK LK LK LK LK LK LK LK
49.	LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK
50.	LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK LK
51.	LR LR LR LR LR LR LR
52.	LR LR LR LR LR LR LR LR LR
53.	LR LR LR LR LR LR LR LR LR LR LR
54.	KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK
55.	KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK KGK
56.	KTK KTK KTK KTK KTK KTK KTK

EXAMPLE 2: ANTIBACTERIAL TESTING

[27] The MIC and IC50 values were determined by a broth microdilution method according to guidelines of the National Committee for Clinical Laboratory Standard as follows: In 96-well tissue culture plates, a fixed volume of bacterial suspension in 2X broth (as defined below) was added to the mixtures or individual compounds dispensed at concentrations varying from 1,000 to 1µg/ml derived from serial two-fold dilutions in sterile water. The bacteria tested were *P. aeruginosa* American Type Culture Collection Number (ATCC) 10145, *E. coli* ATCC 2592, and *S. aureus* (methicillin reseistant) ATTC 33591.

[28] All experiments were compared to bacterial growth under optimal growth conditions (37°C, pH 7.0, absence of additional salt, growth media – referred to as standard growth conditions). In each plate, no growth control (media alone), positive growth control

(bacteria with no test sample), positive antimicrobial controls (agents with known antimicrobial activity) and test compounds were tested. Positive controls included non-periodic antimicrobial peptides D2A21 (FAKKFAKKFKKFAKKFAKFAFAF) and D4E1 (FKLRAKIKVRLRAKIKL).

[29] The plates were incubated overnight, and the relative percent growth determined by optical density at 620 nm (OD620) using a TITERTEK MULTISKAN PLUS™. MIC was defined as the lowest concentration of test sample resulting in 98% growth inhibition. IC50 values were calculated using a sigmoidal curve fitting software program (GRAPHPAD,™ ISI Software,™ San Diego, CA). All samples were tested in duplicate and each assay was repeated at least twice. In the following table, KFAK is the repeat unit and multimers of KFAK were tested. IC50 and MIC are reported in μg/ml.

[30] Table 2. (KFAK)n Anti-microbial Activity Against *P. aeruginosa*:

N	SEQ ID NO	IC50	MIC
D2A21	-	11.3	32-64
1	-	>250	>250
2	-	>250	>250
3	-	>250	>250
4	1	116	>250
5	2	7.7	16-32
6	3	5.6	16-32
7	4	12	16-32

[31] Unexpectedly, these periodic peptides performed as well or better than their unique counterpart (D2A21). Encouraged by this surprising result, a wide variety of periodic peptides were made and tested for anti-bacterial activity using the same protocols. The results are shown below.

[32] Table 3: Periodic Peptides and Anti-Bacterial Activity

Number of Amino Acids	ID	SEQ ID NO	*P aeruginosa*, 10145 gram-		*E. coli 2592* gram-		*S. aureus* (methicillin resistant), 33591 gram+	
			IC50 (μg/ml)	MIC (μg/ml)	IC50 (μg/ml)	MIC (μg/ml)	IC50 (μg/ml)	MIC (μg/ml)
	D2A21	-	11.3	32-62	4.5	8-16	11.9	62-125
	D4E1	-	6.2	8-16	3.3	8-16	6.3	32-62
4	KFAK (1)	-	>500	500	>250	>250	>500	>500
8	KFAK (2)	-	>250	250	>250	>250	>250	>250
12	KFAK (3)	-	>500	500	>250	>250	>500	>500
16	KFAK (4)	1	85	125	46	62-125	>250	>250
20	KFAK (5)	2	5.5	8	6	16-32	>250	>250
24	KFAK (6)	3	5.6	16	4	8-16	38.7	125-250
28	KFAK (7)	4	12.2	16	8	32-62	108.8	>250
32	KFAK (8)	5	19	20	12	16-32	40	125-250
24	RFAR (6)	9	25	32	12	31-62	15	31-62
28	RFAR (7)	10	20	62	8	32-62	31	125-250
32	RFAR (8)	11	30	62	12	32-62	20	125-250
24	FAKK (6)	12	21	62	11	17-32	108	>250
24	AKKF (6)	13	21	25	8	16-32	41	125-250
24	KKFA (6)	14	33	62	7	16-32	136	>250
20	LKKL (5)	15	27	62	49	62-125	69	>250
24	LKKL (6)	16	58	65	31	62-125	98	>250
28	LKKL (7)	17	96	125	41	62-125	252	>250
32	LKKL (8)	18	61	125	37	62-125	80	>250
28	KFAF (7)	19	250	>250	>1000	>1000	>500	>500
28	KFFK (7)	20	87	125	63	>125	133	>250
28	KFAK (7)	21	12.2	16	8	32-62	108.8	>250
28	KAAK (7)	22	>250	>250	261	500-	>500	>500
28	KKAK (7)	23	99	125	26	31-62	60	0
18	KFK (6)	25	74	125	39	62-125	322	>500
21	KFK (7)	26	87	125	18	31-62	141	>500
24	KFK (8)	27	145	250	20	32-62	72	>250
27	KFK (9)	28	68	125	14	16-32	92	125-250
20	KFK (10)	29	31	62	10	12-16	59	>250
18	FKA (6)	35	79	100	29	32-62	>500	>500
21	FKA (7)	36	23	32	7	16-32	>500	>500
24	FKA (8)	37	19	25	7	16-32	74	>500
27	FKA (9)	38	16	20	4	31-62	17	31-62
30	FKA (10)	39	27	32	9	31-62	12	31-62
14	LK (7)	-	111	125	37	45-62	78	250-500
16	LK (8)	44	125	130	65	>125	56	125-250
18	LK (9)	45	74	80	35	62-125	50	250-500
20	LK (10)	46	47	125	127	150-250	29	125-250
22	LK (11)	47	51	62	50	>125	111	>250
24	LK (12)	48	41	45	23	62-125	85	>250

Number of Amino Acids	ID	SEQ ID NO	*P aeruginosa*, 10145 gram-		*E. coli 2592* gram-		*S. aureus* (methicillin resistant), 33591 gram+	
			IC50 (μg/ml)	MIC (μg/ml)	IC50 (μg/ml)	MIC (μg/ml)	IC50 (μg/ml)	MIC (μg/ml)
14	LR (7)	51	109	125	15	31-62	90	250-500
18	LR (9)	52	>250	>250	70	>125	28	125-250
22	LR (11)	53	>250	>250	96	125-250	39	125-250
*PBF16a		-	>250	>250	/	/	/	/
**PBF16b		-	>250	>250	/	/	/	/
***PBF16c		-	>250	>250	/	/	/	/

*PBF16a = KFAKKFAKKFAKKAAK (non-periodic)
**PBF16b = KFAKKFAKKAAKKAAK (non-periodic)
***PBF16c = KFAKKAAKKFAKKAAK (non-periodic)

[33] In reviewing these results, several patterns readily emerge. First, it is clear that periodic peptides made of monomer units as small as a 2mer may be shown to exhibit strong antimicrobial activity (e.g., LK(7-12)). This is very surprising given that most antimicrobial peptides teach that the helix structure must be maintained and employs repeated 7mers to that end and there are very few antimicrobial peptides with beta pleated sheet structures.

[34] Second, its appears that larger peptides have more efficacy than smaller ones. For example, peptides should be at least as big as about 14-16 amino acids to display optimal efficacy (compare KFAK(1-3) versus KFAK(4-8)).

[35] Third, the periodic peptides in many instance demonstrate better activity than the prior art unique peptides (compare D2A21 and D4E1 versus FKAK(5)). This is particularly useful because it means that periodic peptides with equal or better efficacy can be used instead of the prior art unique peptides resulting in substantial cost savings.

[36] Fourth, the tested non-periodic peptides having similar residue content do **not** display antimicrobial activity (see PBF16a-c).

EXAMPLE 3: ANTI-FUNGAL TESTING

[37] Because the antibacterial activity of the periodic peptides was so promising, further experiments were performed to determine if the periodic peptides also had antifungal activity. The experimental design was similar to that above, with

accommodation made for fungal growth requirements, including the use of Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA) slants.

[38] Table 4: Periodic Peptides and Anti-Fungal Activity

Number of Amino Acids	ID	SEQ ID NO	yeast - *C. albicans* - 10231		fungus - *Cr. Neoformans* - 32045	
			IC50 (μg/ml)	MIC (μg/ml)	IC50 (μg/ml)	MIC (μg/ml)
	D2A21	-	121.5	>250	21.9	62-125
	D4E1	-	70.6	80-125	1.8	8-16
4	KFAK (1)	-	329	>500	400	>500
8	KFAK (2)	-	>250	>250	>250	>250
12	KFAK (3)	-	262	280-500	22	125-250
16	KFAK (4)	1	70	90-125	5.3	6.0-8.0
20	KFAK (5)	2	66	90-125	5	8-16
24	KFAK (6)	3	72	80-125	5.8	16-32
28	KFAK (7)	4	82	100-125	9.1	32-62
32	KFAK (8)	5	105	125-250	4.1	8-16
24	RFAR (6)	9	114	125-250	35	62-125
28	RFAR (7)	10	172	250-500	45	62-125
32	RFAR (8)	11	144	250-500	32	62-125
24	FAKK (6)	12	110	125-250	7.6	16-32
24	AKKF (6)	13	225	250-500	11	16-32
24	KKFA (6)	14	107	125-250	12	32-62
20	LKKL (5)	15	211	>500	55	62-125
24	LKKL (6)	16	213	250-500	93	>125
28	LKKL (7)	17	199	250-500	85	100-125
32	LKKL (8)	18	179	250-500	56	62-125
28	KFAF (7)	19	>500	>500	67	125-250
28	KFFK (7)	20	183	250-500	55	62-125
28	KFAK (7)	21	82	100-125	9	32-62
28	KAAK (7)	22	262	>500	41	62-125
28	KKAK (7)	23	>500	>500	28	>63
18	KFK (6)	25	164	250-500	33	40-62
21	KFK (7)	26	175	250-500	26	40-62
24	KFK (8)	27	113	125-250	25	40-62
27	KFK (9)	28	194	250-500	39	62-125
20	KFK (10)	29	104	125-250	30	40-62
18	FKA (6)	35	208	250-500	2.8	4-8
21	FKA (7)	36	203	250-500	3.2	4-8
24	FKA (8)	37	199	250-500	3.9	16-32
27	FKA (9)	38	195	250-500	16	62-125
30	FKA (10)	39	182	250-500	20	32-62
14	LK (7)	-	166	250-500	0.8	2-4
16	LK (8)	44	162	250-500	9.7	32-63
18	LK (9)	45	216	250-500	4.9	32-62
20	LK (10)	46	187	250-500	3.0	62-125
22	LK (11)	47	185	250-500	26	32-62
24	LK (12)	48	175	250-500	22	32-62
14	LR (7)	51	182	250-500	1.8	4-8
18	LR (9)	52	195	250-500	21	62-125
22	LR (11)	53	>500	>500	3.3	62-125

[39] The results show that some of the periodic peptides have highly effective against fungal pathogens (e.g., KFAF(7), KFK(10)), although most are species specific.

EXAMPLE 4: ANTI-TUMOR TESTING

[40] Encouraged by the strong antimicrobial activity of the periodic peptides, tumor cells were also tested. Red blood cells (RBC) were used to ascertain that the periodic peptides would not kill normal mammalian cells, which would obviously limit their effectiveness.

[41] The RBC protocol was from Blondelle (2000) and is described generally as follows: The toxicity toward the HeLa cell line was determined using a MTS (3-(4-5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, sodium salt) cellular reduction assay. MTS (2 mg/mL) was prepared in Dulbecco PBS (pH 7.35), filtered, aliquoted and stored at minus 20°C. In 96 well flat bottomed plates, cell suspensions (250 µL of 6 x 10^4 cell/mL in each well) were incubated for 48 h at 37°C (5% CO2 incubator). The peptides (50 µL) were then added to the cell monolayer (following aspiration of the media from each well and addition of 50 µL of Dulbecco's Modified Eagles Medium) at varying concentrations derived from serial 2-fold dilutions, and the plates incubated for 24 h at 37°C (5% CO2 incubator). Proliferation was then determined by adding a solution of phenazine methosulfate (PMS: 0.92 mg/mL in DPBS) to MTS at a 1:20 ration just prior to the assay. Twenty microliters MTS-PMS solution were added to each well and the plates incubated for 1 h at 37°C (5% CO2 incubator). The relative percent toxicity was determined by comparing the absorbance at 490 nm of each peptide to the absorbance of cells without peptide. The TX50 (concentration required for 50% toxicity) was calculated using a sigmoidal curve fitting software (Graphpad Prism).

[42] Table 5. Periodic Peptides and Anti-Tumor Activity

Number of Amino Acids	ID	SEQ ID NO	RBCs		HeLa	
			TX50 (µg/ml)	% kill at 250 µg/ml	TX50 (µg/ml)	% kill at 500 µg/ml
	D2A21	64	52	93.1	16.9	7.1
	D4E1	65	133.6	77.2	>500	55.2
4	KFAK (1)	57	>500	2.8%	>500	112

8	KFAF (2)	58	>250	2.2%	>500	83
12	KFAK (3)	59	>500	0.4%	>500	77
16	KFAK (4)	1	>250	0.1%	>500	76
20	KFAK (5)	2	>250	4.1%	>500	89
24	KFAK (6)	3	>250	7.3	457.3	30.5
28	KFAK (7)	4	>250	13.7	204.1	13.0
32	KFAK (8)	5	>500	48%	249	18.2%
24	RFAR (6)	9	176	72%	159	22.1%
28	RFAR (7)	10	20	100%	106	12.4%
32	RFAR (8)	11	17	100%	71	9.5%
24	FAKK (6)	12	>500	7%	359	28.3%
24	AKKF (6)	13	39	100%	33	7.3%
24	KKFA (6)	14	>500	2%	>500	76.4%
20	LKKL (5)	15	9	100%	31	-0.7%
24	LKKL (6)	16	11	100%	26	-1.3%
28	LKKL (7)	17	9	100%	25	-0.4%
32	LKKL (8)	18	8	100%	23	0.1%
28	KFAF (7)	19	>500	4%	>500	76.8%
28	KFFK (7)	20	12	100%	30	-0.5%
28	KFAK (7)	21	>250	13.7	204.1	13.0
28	KAAK (7)	22	>500	-6%	>500	90.0%
28	KKAK (7)	23	>500	-6%	>500	>87.9%
18	KFK (6)	25	>500	-7%	>500	77.8%
21	KFK (7)	26	>500	-7%	>500	80.9%
24	KFK (8)	27	>500	-6%	>500	84.2%
27	KFK (9)	28	>500	-6%	>500	80.7%
20	KFK (10)	29	>500	3%	>500	56.8%
18	FKA (6)	35	496	28%	>500	110.8%
21	FKA (7)	36	>500	11%	>500	103.3%
24	FKA (8)	37	>500	10%	>500	60.4%
27	FKA (9)	38	>500	24%	389	20.9%
30	FKA (10	39	>500	51%	234	15.0%
14	LK (7)	60	>500	16%	309	29.6%
16	LK (8)	44	>500	13%	122	43.5%
18	LK (9)	45	>500	21%	137	46.0%
20	LK (10)	46	>500	27%	>500	65.0%
22	LK (11)	47	>500	24%	202	43.8%
24	LK (12)	48	>500	35%	49	60.0%
14	LR (7)	51	>500	15%	>500	86.5%
18	LR (9)	52	>500	24%	>500	96.7%
22	LR (11)	53	450	41%	>500	66.6%

[43] As expected, some of the periodic peptides have anti-tumor activity, but do not destroy normal cells such as red blood cells (RBCs). In particular, the dipeptides LK(8-9, 12) appear very promising; killing HeLa cells, but not RBCs. However, anti-tumor

activity is less predictable than antimicrobial activity, and each periodic peptide should be tested against a range of cells before use.

EXAMPLE 5: BIOCIDAL TESTING

[44] In the previous experiment, IC50 and MIC were measured because these are simple, common tests that are easy to perform. However, these tests actually measure **biostat** activity, and not true **biocidal** activity. Thus, biocidal activity was measured in this example.

[45] Biocide efficacy protocols were improved by reducing sample size, organizing the test material into an array format, implementing most probable number (MPN) quantitation and using multi-channel liquid handling equipment. We call the new method "high-throughput microanalysis and rapid quantitation" or "HMARQ." HMARQ is directly applicable to current industrial efficacy tests such as multi-cycle preservation challenge or time course disinfection tests.

[46] HMARQ is performed in a high throughput plate, such as 96-well microtiter plates. Typical sample volumes have been reduced down to 200 to 300 μL, but can be reduced further if desired. In these samples, no more than 10% of the total volume will be composed of the biocide and organism solution, and all non-matrix additions are normalized for all samples.

[47] The sample matrix is first inoculated with the desired concentration of microorganisms. Inoculated sample matrix is then added to the 96-well assay block containing the biocide(s) under study. Each sample block contains biocide treated samples and untreated control samples (lacking biocide). Once the samples are prepared, the entire block of samples is mixed by vortexing until each sample is homogenous. In general, the study starts once mixing is complete, and samples are removed as required for the analysis. When Kill Time testing (rate of biocide activity) is performed, the microorganisms are added after biocide addition to the samples, allowing for rapid mixing and analysis.

[48] Bacterial concentration (CFU/mL) is determined using the most probable number method (MPN). The contaminated solution is serially diluted until the "no growth" endpoint is reached. The endpoint represents the MPN and is expressed in units of the

bacterial concentration. A serial 1:10 dilution will yield a bacterial concentration resolution of 1 log and the log reduction is determined by comparing the concentration of organisms in a treated sample to the concentration of organisms in untreated samples. For example, if a sample requires four 1:10 dilutions before bacterial growth is lost then the MPN for bacterial concentration in the sample is less than or equal to 1 X 10^4 CFU/mL (1E4). If each well in a serial 8-fold dilution shows bacterial growth, then the MPN is greater than or equal to 1 X 10^8 CFU/mL (1E8). This method of enumeration is generally applicable to all non-filamentous microorganisms.

[49] Media included Tryptic Soy Broth (TSB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi. These are available commercially and prepared according to the manufacturers instructions. Indicator medium was TSB/R for bacteria and SDB/R for fungi. These were made by addition of 50 μM filter sterilized Resazurin to the sterilized and cooled medium. Tryptic Soy agar plates (TSA) and SDA slants were used to provide inoculant for bacterial and fungal cultures. The indicator dye appeared pink or white when bacterial growth was present. Blue indicated no growth and purple indicated that growth was present and would resolve with additional time.

[50] Using the HMARQ test described above, periodic peptides were generated and tested for Kill Activity. In this experiment, a broader range of peptidezs were tested for activity. Units are expressed as ppm (μg/mL) needed for 3.5 log kill at 15 min or 24 hour and the results are shown below.

[51] Table 6: Periodic Peptides and Biocidal Activity

Number of Amino Acids	ID	SEQ ID NO	*P. aeruginosa* ATCC 15442 (gram -)		*Staph. aureus* ATCC 6538 (gram +)	
			15 min	24 hr	15 min	24 hr
32	KFAK(8)	5	31	31	>500	8
36	KFAK(12)	6	16	31	31(3 log)	>500
52	KFAK(17)	7	16	250	250	500
80	KFAK(20)	-	8	16	4	4
28	KKAK(7)	23	16	16	>500	125
28	KFAF(7)	19	63	63	>500	125
24	RFAR(6)	9	31	31	>500	8
28	RFAR(7)	10	63	125	>500	4
32	RFAR(8)	11	>500	16	>500	125
24	FAKK(6)	12	>500	16	>500	250
24	AKKF(6)	13	>500	16	>500	125
24	KKFA(6)	14	>500	8	>500	63

Number of Amino Acids	ID	SEQ ID NO	*P. aeruginosa* ATCC 15442 (gram -)		*Staph. aureus* ATCC 6538 (gram +)	
			15 min	24 hr	15 min	24 hr
20	LKKL(5)	15	>500	8	>500	125
24	LKKL(6)	16	>500	8	4	125
28	LKKL(7)	17	125	8	4	125
32	LKKL(8)	18	4	8	>500	125
28	KFFK(7)	20	16	8	4	63
28	KAAK(7)	22	>500	125	>500	250
9	KFK(3)	-	>500	125	>500	>500
12	KFK(4)	-	500	125	>500	>500
18	KFK(6)	25	>500	125	>500	
21	KFK(7)	26	>500	125	>500	
24	KFK(8)	27	>500	>125	>500	500
27	KFK(9)	28	>500	63	>500	500
30	KFK(10)	29	8	8	4	500
36	KFK(12)	30	4	8	>500	250
48	KFK(16)	31	16	31	>500	16
63	KFK(21)	32	31	63	>500	250
6	FKA(2)	33	>500	125	16	500
12	FKA(4)	34	>500	125	4	250
18	FKA(6)	35	8	8	4	500
21	FKA(7)	36	125	63	500	500
24	FKA(8)	37	125	16	8	125
27	FKA(9)	38	4	4	8	125
30	FKA(10)	39	8	16	8	8
51	FKA(17)	40	16	16	4	8
63	FKA(21)	41	8	16	4	125
4	LK(2)	42	>500	250	>500	16
10	LK(5)	43	62	62	>500	31
14	LK(7)	-	8	8	16	4
16	LK(8)	44	8	16	31	4
18	LK(9)	45	8	8	16	4
20	LK(10)	46	8	8, 166	16 or less	16 or less
22	LK(11)	47	16	8	16 or less	4
24	LK(12)	48	16 (3 log)	4	4	8
36	LK(18)	49	16	31	8	16
48	LK(24)	50	16	63	>500	>500
14	LR(7)	51	>63	4	31	7.8
18	LR(9)	52	31(3 log)	4	16	8
22	LR(11)	53		8	16	8
21	KGK(7)	-	500	500	>500	500
33	KGK(11)	54	8	8	>500	8
45	KGK(15)	55	4	16	125	62
21	KTK(7)	56	31	31	250	8

[52] The results indicate that many of the periodic peptides have true biocidal activity. Surprisingly, even peptides too small to span the membrane demonstrate biocidal activity (FKA(2), FKA(4), LK(2), LK(4)). Therefore, the lower size limit for periodic peptides can in fact be as low as 4 residues.

EXAMPLE 6: VIRAL TESTING

[53] The prior experiments established bactericidal and fungicidal activity, as well as anti-tumor activity. The next prophetic experiments will establish antiviral activity.

[54] Unique antimicrobial peptide D2A21, which has amino acid content similar to the peptides demonstrated herein, has been shown to have anti-bacterial, antifungal, anti-tumor and antiviral activity. Similarly, two well characterized natural antimicrobial peptides from insects - melittin and cecropin - have been shown to be effective against human immunodeficiency virus 1 (HIV-1), with IC50 values in the range 0.9-1.5 mM for melittin and 2-3 mM for cecropin (Wachinger (1998)). Therefore, we predict that a subset of the peptides described above will also have antiviral activity.

[55] Antiviral activity can be measured in a number of ways, but one simple method of determining the effect on a retrovirus, such as HIV or FIV, is to measure decreased reverse transcriptase (RT) activity of a retrovirus and determine the 50% inhibitory concentration, which should be about 1 mM for effectiveness (Jia Ma (2002)).

[56] All references cited herein are incorporated by reference in their entirety. The references are listed herein for convenience:

1. Durell SR, et al., *Modeling the ion channel structure of cecropin*, Biophys J. (1992 Dec) 63(6):1623-31.

2. E. Gazit, et al., *Interaction of the Mammalian Antibacterial Peptide Cecropin P1 with Phospholipid Vesicles*,. Biochemistry (1995) 34, 11479.

3. Arlotti *et al., Efficacy of a synthetic lytic peptide in the treatment of prostate cancer*, Urol Oncol. (2001) 6(3): 97-102.

4. US5789542

5. Javadpour, *et al., De Novo Antimicrobial Peptides with Low Mammalian Cell Toxicity*, J. Med. Chem. (1996) 39(16): 3107-3113.

6. Wachinger, *et al., Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression,* J. Gen. Virol. (1998) (79): 731-740.

7. Jia Ma, et al., *Inhibitory Activity of Synthetic Peptide Antibiotics on Feline Immunodeficiency Virus Infectivity In Vitro*, J. Virol. (2002) 76(19): 9952-9961.

8. S.E. Blondelle and Karl Lohner, *Biopolymers (Peptide Science),* (2000) Vol 55, 74- 87.